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Synthesis and evaluation of new elastase inhibitors. I. 1,1-Dioxocephem-4-thiolesters

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Summary — Several 7α-chloro and 7α-methoxy cephalosporin thiolester sulphones variously substituted at the C-3' position were synthesized from 7-amino-3-deacetoxycephalosporanic acid (7-ADCA). The compounds are time-dependent inhibitors of human leukocyte elastase (HLE) with effective K_i ranging from micro- to nanomolar values, and second order rate constant reaching 10^6 $M^{-1}s^{-1}$; they also inhibit porcine pancreatic elastase with similar, though not identical efficiency. Compared to the corresponding cephem esters, the thiolesters of the 7-Cl series inhibit HLE up to 10 times as fast. Complete enzyme inactivation is achieved when a leaving group at C-3' (OAc or S-Het) is present, at the expense however of stability towards hydrolytic β-lactam cleavage. In the 7-OMe series the thiolester compounds are far more stable and retain good efficiency for irreversible enzyme inhibition, superior to that displayed by the corresponding ester compounds. Three representative compounds (including one ester and two thiolesters) are shown to be effective inhibitors of HLE in the presence of insoluble elastin.

cephalosporin sulphones / thiolesters / enzyme inhibition / human leukocyte elastase / porcine pancreatic elastase

Introduction

In 1986, a communication from Merck, Sharp and Dohme [1] revealed that cephalosporin antibiotics can be modified to elicit potent inhibitory activity against human leukocyte elastase (HLE, EC 3.4.21.37), a serine endopeptidase implicated in the pathogenesis of pulmonary emphysema and other connective tissue diseases [2]. Since then, much work has been reported in this area. Considerable chemical efforts were addressed to the modification of the C-7 [3], C-2 [4] and C-3 [5] position of the cephalosporin moiety. Studies at the C-4 position, limited to the evaluation of esters and amides [6], were more conservative.

Antibacterial cephalosporins are thought to be substrate analogs of the D-alanyl-D-alanine end of peptidoglycan strands which are to be cross-linked to one another in the growing bacterial cell wall; the β-lactam CO-N bond corresponds to the scissile peptide bond involved in the transpeptidation reaction catalyzed by the target enzymes and the C-4 carboxyl group corresponds to the carboxyl of the terminal D-alanine. Accordingly, modification of the C-4 carboxyl of cephalosporins prevents enzymic recognition and suppresses antibacterial activity. Study of cephalosporins as elastase inhibitors opens up new possibilities since HLE, as opposed to the bacterial enzymes, is an endopeptidase. The C-4 substituent can be omitted altogether [1, 6]. Even better, it can be formulated in such a way as to improve initial binding [3–6]. Additionally, it should provide a good compromise between overall hydrolytic stability of the molecule and reactivity of the β-lactam towards the catalytic Ser-195 hydroxyl of HLE. Ideally, it should assist the occurrence of a second inactivating event [7], whereby enzymic inhibition becomes persistent.

On these grounds, a research programme devoted to the synthesis and evaluation of novel C-4 substituted cephem sulphones was undertaken in our laboratories. We wish here to report preliminary results

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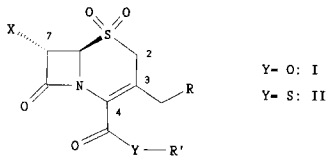
obtained in the class of cephalosporin thiolesters **II**, which were first selected as readily accessible bioisosteres of the active ester counterparts **I** reported by MSD research laboratories [1, 3, 5].



Chemistry

Our first concern was to find reliable thioesterification and esterification procedures that would allow the preparation of the target products **II** and reference compounds **I** from few common intermediates, *ie* the 4-carboxycephem 1,1-dioxides **12** (scheme 1), **15** (schemes 1, 3, 4), **18** (scheme 2), and **22** (scheme 5). The choice of delaying the esterification/thioesterification at the end of the synthesis provided the desired versatility and bore benefits related to the deaminative functionalization at C-7 of 7-aminocephalosporanic acid (7-ACA) and 7-amino-3-deacetoxy-cephalosporanic acid (7-ADCA), which can be run conveniently without protection of the carboxyl group. Diazotization of 7-ADCA, in particular, was economically and safely performed with nitrous acid and led, according to the conditions, to the 7α-chloro or 7α-methoxy derivative. Oxidation of the crude products with potassium peroxymonosulphate (Oxone) gave the key intermediates **12** and **22**. The advantages of this strategy as an entry to 7-modified cephem sulphones, and details on the synthesis of the 7α-methoxy compound **22**, is the subject of a separate communication [8].

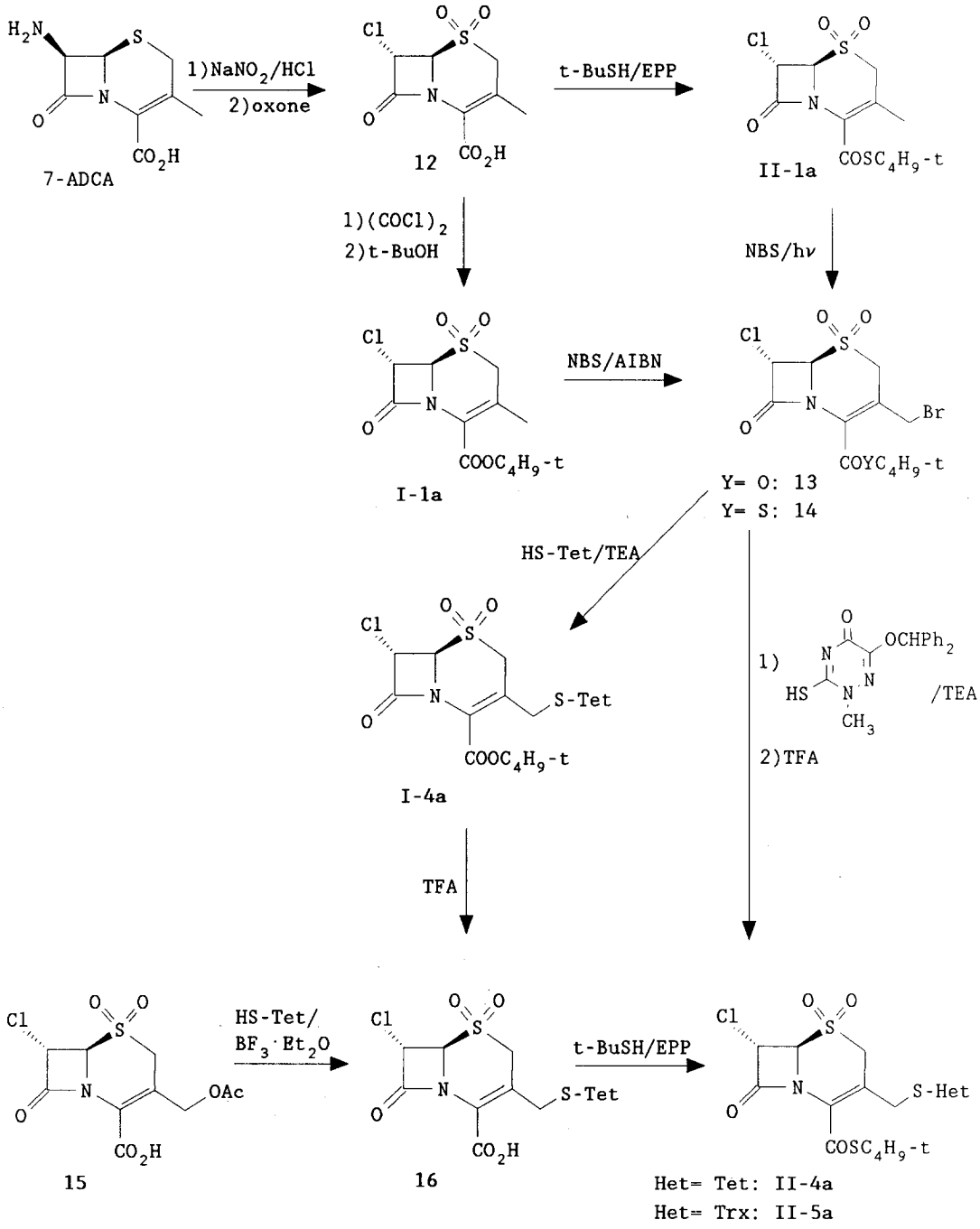
For the synthesis of esters **I**, activation of the cephem carboxyl group as the acid chloride under Vilsmeier conditions was preferred. On usual cephalosporin substrates, this method is known to lead to the biologically inactive Δ^2-isomers, presumably *via* a conjugated cephem 4-ketene [9]. The cephalosporin sulphones previously described as HLE inhibitors were obtained by esterification (at the sulphide oxidation level) with *N,N'*-diisopropyl-*O*-alkylisoureas or by treatment with diazoalkanes [6]. We found the acid chloride method, performed at the sulphone oxidation level *(Experimental protocol A)*, exceedingly convenient and versatile.

For the synthesis of the thiolesters **II** from the corresponding 4-carboxycephem 1,1-dioxides, several possibilities were examined. The acid chloride method, using mercaptans in place of alcohol, proved unsatisfactory. Condensation of the carboxylic acid and the thiol in the presence of 1,3-dicyclohexylcarbodiimide (DCC), recently proposed as a convenient procedure for thiolester synthesis [10], was impaired by the competitive formation of 4-spiroazetidinyl cephems [11], *eg* **19** (scheme 3). The *n*-butyl thiolester **II–3d** was the only compound prepared by this method; with hindered thiols the spiroazetidinyl product was exclusively obtained. Finally, freshly prepared polyphosphate ester (EPP) [12] was found to be a convenient condensing agent *(Experimental protocol B)*.

The criteria and methodologies mentioned above were utilised in the preparation of the cephem sulphones described in this paper, which are best examined according to their C-7 and C-3 substituents (schemes 1–5). The results are summarized in table I, which shows the obtained products, the pertinent scheme, the closest purified precursors and the isolated yields based on the latter.

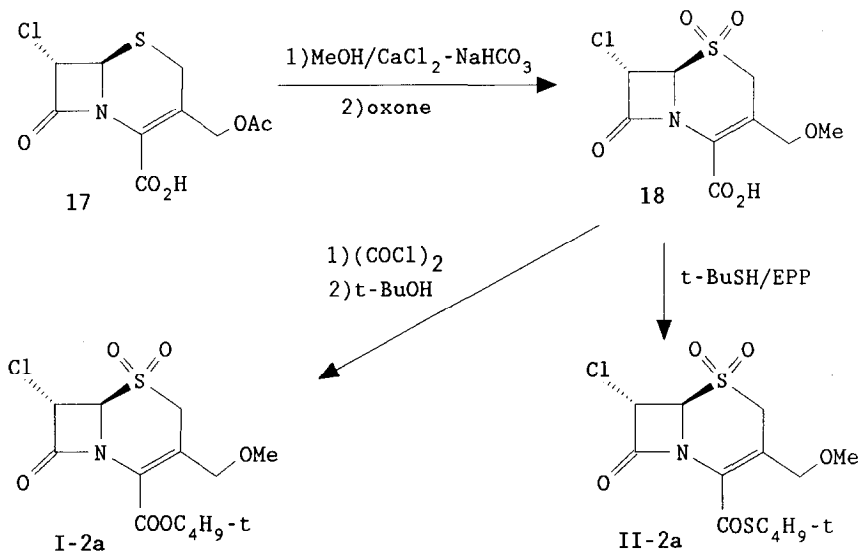
The 7α-chloro-3-methyl and 7α-chloro-3-(heterocyclyl)thiomethyl compounds are represented in scheme 1. The former compounds, ester **I–1a** and thiolester **II–1a**, were obtained from crude **12** by *Protocols A* and *B*. The latter compounds, ester **I–4a** and thiolesters **II–4a**, **II–5a**, were addressed starting from obtained 3-methylcephem sulphones. Bromination of the C-3 methyl of cephem sulphones [13] with *N*-bromosuccinimide (NBS) in the presence of azobisisobutyronitrile (AIBN) as a radical initiator *(Experimental protocol C)* worked well on the *tert*-butyl esters **I–1a** (scheme 1) and **I–6a** (scheme 5). The same reaction failed when run on the thiolester **II–1a**, but the desired 3-bromomethylcephem intermediate **14** was generated, albeit in poor yield, under photochemical conditions [13, 14]. Compound **13** underwent smooth displacement by 5-mercapto-1-methyl-1,2,3,4-tetrazole in the presence of triethylamine to afford **I–4a**. In a similar manner, **14** reacted with 6-benzhydryloxy-2,5-dihydro-3-mercapto-2-methyl-5-oxo-1,2,4-triazine [15], providing, after removal of the benzhydryl group with trifluoroacetic acid (TFA), the *tert*-butyl thiolester **II–5a**. Owing to the poor yields of the photoinitiated bromination, an alternative route was preferred for the synthesis of the C-3'-tetrazolyl analogue **II–4a**. Accordingly, the free-acid precursor **16** was prepared, either by conventional hydrolysis of the *tert*-butyl ester **I–4a** with TFA *(Experimental protocol D)* or, more expeditiously, by Lewis acid catalyzed displacement of the cephalosporanic sulphone **15** with 5-mercapto-1-methyl-1,2,3,4-tetrazole. Condensation of **16** with *tert*-butyl mercaptan by the EPP method to give **II–4a** was smooth as usual.

Scheme 2 outlines the synthesis of cephems bearing a methoxymethyl group at C-3. The key step is the displacement with methanol of the C-3' acetoxy

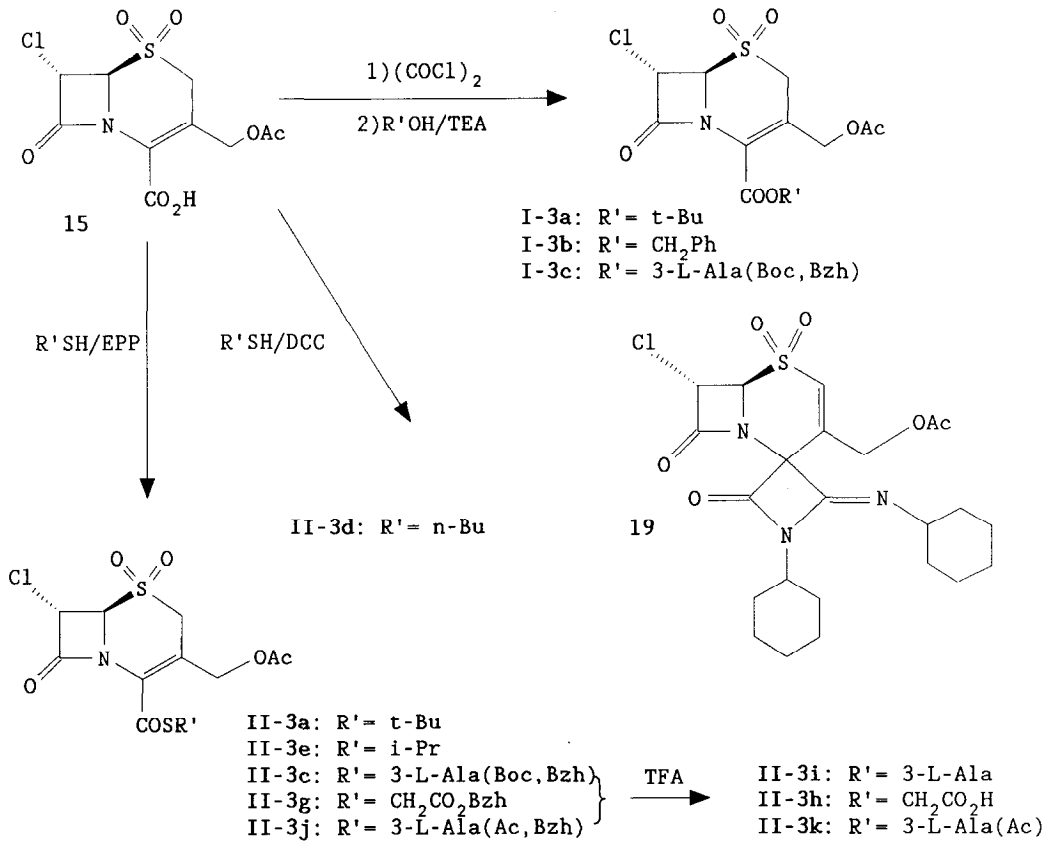


Scheme 1. Synthesis of 7α-chloro-3-methyl and 7α-chloro-3-(heterocyclyl)-thiomethyl compounds. EPP: ethyl polyphosphate; NBS: *N*-bromosuccinimide; AIBN: azo-bis-isobutyronitrile; TEA: triethylamine; TFA: trifluoroacetic acid; Tet: 1-methyl-1,2,3,4-tetrazol-5-yl; Trx: 2,5-dihydro-6-hydroxy-2-methyl-5-oxo-1,2,4-triazin-3-yl.

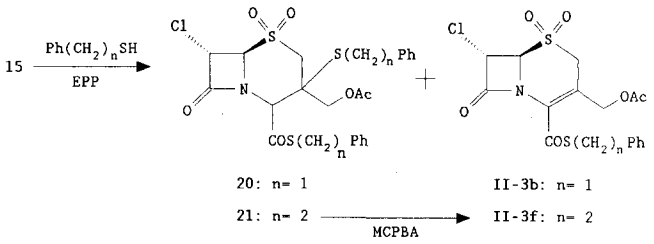
group. This reaction was run on the 7α-chloro-cephalosporanic acid **17** [11, 16] in the presence of sodium hydrogen carbonate and a large excess of calcium chloride, as previously described in the literature [17]. The crude product was oxidized with oxone to afford sulphone **18**, which was easily converted to the *tert*-butyl ester **I–2a** and *tert*-butyl thiolester **II–2a** according to the general protocols.



Scheme 2. Synthesis of 7α-chloro-3-methoxymethylcephem compounds.

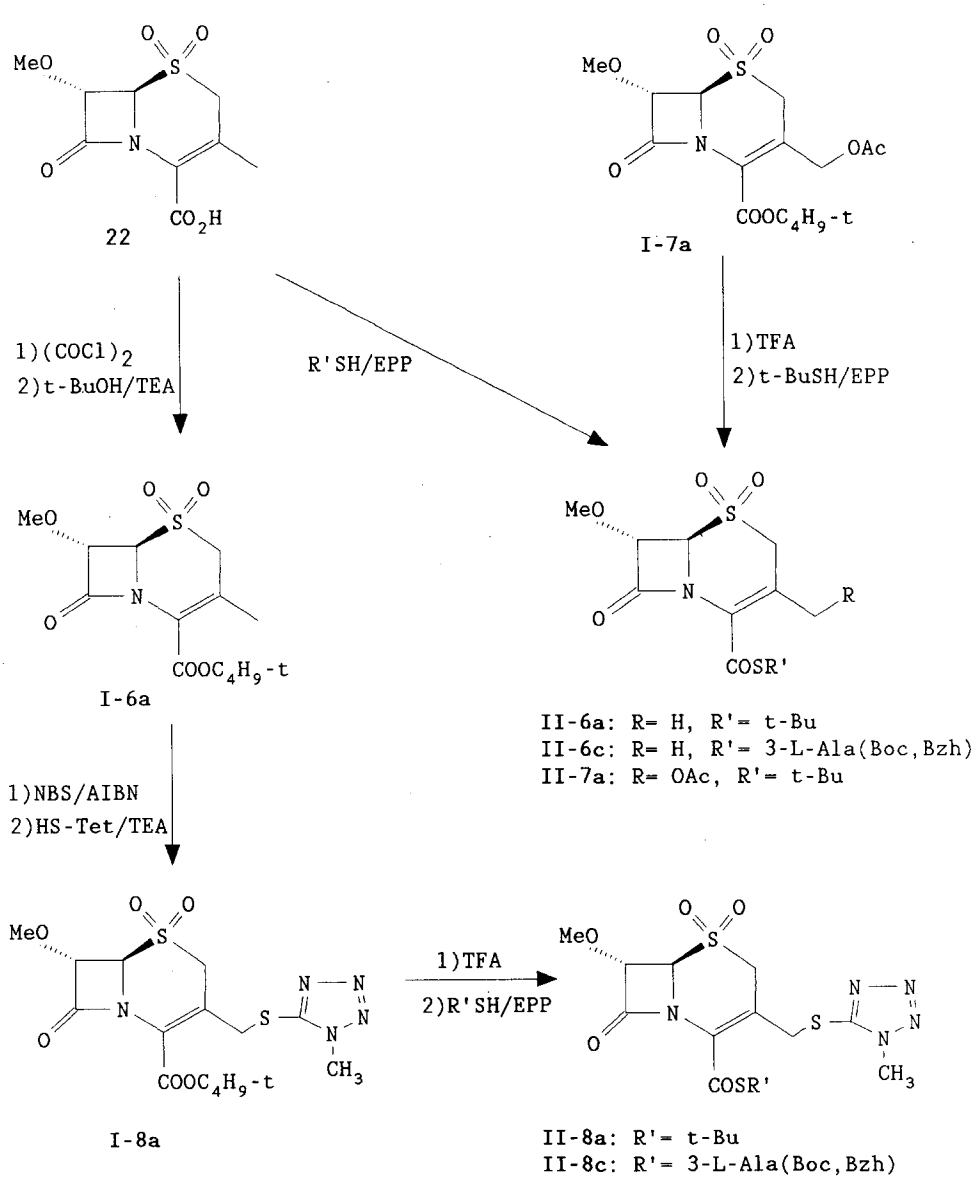


Scheme 3. Synthesis of 7α-chloro-3-acetoxymethylcephem compounds. For abbreviations and acronyms see scheme 1 (legend) and table I (footnotes).



Scheme 4. Addition and elimination reactions at the C-3/C-4 position of benzyl and phenethyl cephem thiolesters.

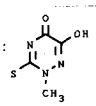
The 7-chlorocephalosporanic sulphone **15** served as the immediate precursor of the 7α-chloro-3-acetoxymethylcephem esters **I–3a**, **I–3b**, **I–3c** and thiolesters **II–3a**, **II–3c**, **II–3d**, **II–3e**, **II–3g**, **II–3j** (scheme 3). Removal of the acid-labile groups from **II–3c**, **II–3g**, **II–3j** with TFA gave the water-soluble cysteine and thioglycolic derivatives **II–3i**, **II–3h**, **II–3k**. Unexpectedly, condensation of **15** with benzyl and phenethyl mercaptan (scheme 4) proceeded further to give the Michael adducts **20** (isolated together with the desired product **II–3b**) and **21** (instead of **II–3f**). It is

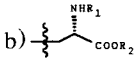


Scheme 5. Synthesis of 7α-methoxycephem compounds.

Table I. Cephem sulphones esters **I** and thiolesters **II**.

Compd No	*X*	*R*[a]	*R*[b]	*Starting material (scheme of synthesis)*		*Yield (%)*
I–1a				**7-ADCA**	(Scheme 1)	33
II–1a	Cl	H	*t*-Bu	**12**	(Scheme 1)	53
I–2a				**17**	(Scheme 2)	14
II–2a	Cl	OMe	*t*-Bu	**18**	(Scheme 2)	39
I–3a				**15**	(Scheme 3)	63
II–3a	Cl	OAc	*t*-Bu	**15**	(Scheme 3)	73
I–3b				**15**	(Scheme 3)	71
II–3b	Cl	OAc	CH_2Ph	**15**	(Scheme 4)	31
I–3c				**15**	(Scheme 3)	41
II–3c	Cl	OAc	3-L-Ala(Boc, Bzh)	**15**	(Scheme 3)	73
II–3d	Cl	OAc	*n*-Bu	**15**	(Scheme 3)	23
II–3e	Cl	OAc	*i*-Pr	**15**	(Scheme 3)	79
II–3f	Cl	OAc	CH_2CH_2Ph	**21**	(Scheme 4)	93
II–3g	Cl	OAc	CH_2CO_2Bzh	**15**	(Scheme 3)	75
II–3h	Cl	OAc	CH_2CO_2H	**II–3g**	(Scheme 3)	83
II–3i	Cl	OAc	3-L-Ala	**II–3c**	(Scheme 3)	53
II–3j	Cl	OAc	3-L-Ala(Ac, Bzh)	**15**	(Scheme 3)	39
II–3k	Cl	OAc	3-L-Ala(Ac)	**II–3j**	(Scheme 3)	85
I–4a				**I–1a**	(Scheme 1)	41
II–4a	Cl	S-Tet	*t*-Bu	**I–4a**	(Scheme 1)	72
II–5a	Cl	S-Trx	*t*-Bu	**II–1a**	(Scheme 1)	3
I–6a				**22**	(Scheme 5)	64
II–6a	OMe	H	*t*-Bu	**22**	(Scheme 5)	73
II–6c	OMe	H	3-L-Ala(Boc, Bzh)	**22**	(Scheme 5)	75
I–7a				**7-ACA**	[20]	20
II–7a	OMe	OAc	*t*-Bu	**I–7a**	(Scheme 5)	53
I–8a				**I–6a**	(Scheme 5)	29
II–8a	OMe	S-Tet	*t*-Bu	**I–8a**	(Scheme 5)	43
II–8c	OMe	S-Tet	3-L-Ala(Boc, Bzh)	**I–8a**	(Scheme 5)	54

a) OAc: $OCOCH_3$; S-Tet:  ; S-Trx: 

b) 

3-L-Ala : $R_1 = R_2 = H$
3-L-Ala(Ac): $R_1 = COCH_3$, $R_2 = H$
3-L-Ala(Ac,Bzh): $R_1 = COCH_3$, $R_2 = CHPh_2$
3-L-Ala(Boc,Bzh): $R_1 = CO_2t\text{-}Bu$, $R_2 = CHPh_2$

interesting to recall that the 2,3-double bond of cephems is quite resistant to nucleophilic reagents [18] and addition of mercaptans has only intramolecular precedents [19]. The temporal sequence of the reaction was ascertained by exposing the isolated thiolester **II–3b** to benzyl mercaptan (acetonitrile, pyridine catalysis). Smooth addition occurred, affording **20** almost quantitatively within a few hours; by contrast, no reaction took place on the acid **15** and the ester **I–3b**. Clearly, the thiolester moiety at the C-4 position provides the cephem acrylic moiety with an extra-activation towards nucleophiles. In order to obtain **II–3f**, the adduct **21** was exposed to one molar equivalent of *m*-chloroperoxybenzoic acid (MCPBA) in the cold. As expected, oxidation regioselectively occurred at the alkyl sulphur atom. Loss of benzylsulphenic acid spontaneously occurred during work-up, providing **II–3f** in excellent yield.

The preparation of 7α-methoxycephem compounds is outlined in scheme 5. The 7α-methoxy-3-deacetoxycephalosporanic acid sulphone **22**, efficiently prepared from 7-ADCA by a two-step procedure developed in our laboratories [8], served the synthesis of the 3-methylcephems **I–6a**, **II–6a**, **II–6c** and the 3-tetrazolylthiomethyl derivatives **I–8a**, **II–8a**, **II–8c**. The former compounds were prepared according to the general esterification and thio-esterification protocols, while the latter were prepared starting from obtained **I–6a** according to the bromination/displacement procedure detailed above. The resulting *tert*-butyl ester **I–8a** was converted to thiolesters **II–8a**, **II–8c** by sequential hydrolysis and thioesterifications *(Protocols D* and *B)*; similarly, the cephalosporanic ester **I–7a** [20] was converted to the corresponding thiolester **II–7a**. Previously, the tetrazolylthiomethylcephem **I–8a** had been prepared by an elaborate sequence entailing hydrolysis of the cephem C-3' acetoxy with titanium (IV) isopropoxide which, admittedly, is not compatible with ester derivatives other than the *tert*-butyl [5].

Enzymology

Kinetics of HLE inhibition

When catalytic amounts of HLE are rapidly mixed with a substrate solution containing an appropriate concentration of any of the inhibitors in table II, product formation, which is linear in the absence of inhibitors, exponentially decreases in time until a new steady state linear phase is obtained. Experimental data are generally interpretable with equation 1, which has been derived for the case of slow-binding reversible inhibitors [21].

$$[P] = (v_0 - v_s)\,(1 - e^{-kt})/k + v_s t \qquad [1]$$

v_0 = initial reaction velocity
v_s = reaction velocity at steady state
k = apparent first order rate constant

It is, however, inappropriate to consider cephalosporin sulphones as reversible inhibitors, since all other experimental evidence [3, 6] points to their nature as irreversible suicide substrates or mechanism-based inhibitors (scheme 6) [22]. In fact, it has been shown [3] that incubation of HLE with an excess of **I–3a** leads to complete inhibition which is not reversed upon dilution with substrate. Furthermore, the chemical mechanism of inhibition, as inferred from crystallographic data of an adduct between **I–3a** and PPE [7], involves the β-lactam ring opening by the catalytic Ser residue, and then a series of chemical events leading to the formation of either a new bond or a non-covalent interaction between the reacted inhibitor molecule and the His residue of the enzyme catalytic triad [3]. In keeping with this proposed double hit mechanism, we have found that compounds lacking a leaving group at the 3'-position (**I–1a**, **II–1a**, **I–6a**, **II–6a**) are completely hydrolysed by HLE, though at a slow rate, and thus behave as poor substrates rather than inhibitors. On the other hand, inhibitors **I–3a**, **II–3a**, **I–7a**, **II–7a**, when incubated with micromolar HLE for a moderately long time (30 min at 37°C), produce linear plots of residual enzyme activity as a function of inhibitor concentration. These plots intercept the *x*-axis at a finite inhibitor concentration (I_0) whereafter no detectable enzyme activity remains. The ratio $r = I_0/E_t$, where E_t is the total enzyme concentration, is a measure of the mol of inhibitor used for complete inhibition of one mol of enzyme. This ratio is slightly above one (1.5–1.6) for compounds **I–3a**, **II–3a** and **II–7a**, whereas a significantly higher value is observed for **I–7a** (2.7 ± 0.2). For mechanism based inhibitors $r = 1 + k_3/k_4$, and therefore $r - 1$ measures the efficiency of side reactions leading to recovery of enzyme activity in comparison to that of the enzyme inactivation process. Spontaneous hydrolysis is yet another process (not shown in scheme 6) which may be responsible for *r*-values exceeding unity, as measured for instance with compounds **I–3a** and **II–3a**. However, the results obtained for inhibitors **I–7a** and **II–7a**, which both have half-lives of several hours, must be interpreted as a reliable measure of competition between the two final branches in scheme 6, and increased efficiency for irreversible enzyme inhibition is thus observed in the case of the thiolester compound.

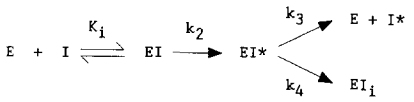
Solutions to the kinetic mechanism of scheme 6 have been proposed at various levels of approximation [23, 24], and deviations from first order kinetics are expected. This complex time dependence, however, may be difficult to detect experimentally within a

Table II. Inhibition parameters (PPE, HLE) and hydrolytic stability ($t_{1/2}$ at pH 7.4, 37°C) of 1,1-dioxocephem esters **I** and thiolesters **II**[a].

Compound	*PPE* IC_{50} *(μg/ml)*	k_{on} $(M^{-1} s^{-1})$	*HLE* k_2/K_i $(M^{-1} s^{-1})$	K_i *(ss)* [b] *(nM)*	$t_{1/2}$ *(h)*
7α-Chlorocephem esters					
I–1a	3.0	1.5×10^3		360	5.1
I–2a	0.15		1.8×10^4	80	1.7
I–3a[c]	0.01	1.5×10^5		5	1.3
I–3b	0.005	1.9×10^5		(17)	0.7
I–3c	ND[d]	4.8×10^5		(8)	0.6
I–4a	0.012	1.5×10^5		3	1.2
7α-Chlorocephem thiolesters					
II–1a	1.5	1.8×10^4		100	2.1
II–2a	0.05		3.0×10^5	16	1.1
II–3a	0.03	6.4×10^5		3	0.6
II–3b	< 0.01	2.1×10^6		(7)	0.8
II–3c	0.02	2.0×10^5		(16)	1.9
II–3d	0.015	1.7×10^6		(2)	0.6
II–3e	0.03	3.0×10^5		(5)	0.5
II–3f	0.02	2.0×10^6		(2)	0.7
II–3g	0.02	9.1×10^5		(6)	1.4
II–3h	0.3	ND		ND	0.5
II–3i	1.0	ND		ND	0.08
II–3j	ND	6.4×10^5		(8)	1.7
II–3k	ND	4.2×10^4		60	0.5
II–4a	0.015		1.0×10^6	4	0.7
II–5a	ND	3.9×10^5		(5)	1.0
7α-Methoxycephem esters and thiolesters					
I–6a	ND	$< 1.0 \times 10^2$		5400	40
I–7a[e]	0.02	1.1×10^4		18	3.2
I–8a[f]	0.06	2.0×10^4		13	6.6
II–6a	ND		1.9×10^3	630	58
II–6c	0.5	1.1×10^4		70	12.4
II–7a	ND	1.4×10^5		(4)	7.0
II–8a	0.08		9.6×10^4	(7)	8.0
II–8c	0.03		4.6×10^5	(8)	6.5

[a]Values are rounded to the last significant figure. Generally, SDs are within 1–5% of each given value, but in a few exceptions a 10% deviation has been tolerated; [b]values obtained by analysis of steady state velocity data with eq 3. For the most potent inhibitors, systematic deviations from fitting equations have been observed; the corresponding values are given in brackets. These deviations are attributed to the irreversible nature of enzyme–inhibitor interaction; [c]Lit [3]: $k_{on} = 1.61 \times 10^5$ M^{-1} s^{-1}; [d]ND: not determined; [e]Lit [3, 5]: $k_{on} = 1.27 \sim 1.9 \times 10^4$ M^{-1} s^{-1}; [f]Lit [5]: $k_{on} = 1.5 \times 10^4$ M^{-1} s^{-1}.

limited observation time window, and at its early stages can hardly be distinguished from reversible slow binding inhibition. Because a complete investigation into the kinetics of HLE inhibition by cephem sulphones is out of the scope of the present study, we have adopted an empirical approach, which is consistent with that used by others [4]. Within this framework, apparent first order rate constants, k, should depend on inhibitor concentration according to eq 2a, which properly takes into account the equilibrium binding step of enzyme and inhibitor. This is observed only in a few cases; more often a linear dependence is found (eq 2b), as expected for a one-step equilibrium mechanism (fig 1).



Scheme 6. Kinetic scheme of HLE inhibition by cephem sulphones. E: enzyme; I: inhibitor; EI: Michaelis complex; EI*: acylenzyme intermediate; I*: inactivated inhibitor; EI_i: inactivated enzyme–inhibitor complex.

$$k = k_{off} + k_2/K_i \cdot \frac{[I]}{1 + [S]/K_m + [I]/K_i} \quad [2a]$$

$$k = k_{off} + k_{on} \cdot \frac{[I]}{1 + [S]/K_m} \quad [2b]$$

With all likelihood, linear dependence is a consequence of values of K_i being high in comparison to the accessible range of inhibitor concentration, as eq 2a reduces to eq 2b with $k_{on} = k_2/K_i$ for $K_i \gg [I]$. For the sake of comparison, we report in table II the parameters k_2/K_i or k_{on}, respectively. Values of k_{off} are largely inaccurate and are not reported. This originates from their values being close to or lower than our experimental detection limit (about 1×10^{-3} s^{-1}), which is dictated by other concomitant effects (enzyme inactivation, substrate depletion, photobleaching of the released aminocoumarin, etc).

The dependence of steady state velocity, v_s, on inhibitor concentration was analysed with eq 3, the ordinary hyperbolic law for competitive inhibition, where v_u is the velocity of the reaction in the absence of inhibitor.

$$v_s = v_u \cdot \frac{1 + [S]/K_m}{1 + [S]/K_m + [I]/K_i(ss)} \quad [3]$$

In view of the irreversible nature of the inhibition process, values of $K_i(ss)$ indicate relative potency of each inhibitor in an empirical sense rather than in their ordinary meaning of dissociation equilibrium constants. Moreover, systematic deviation is observed in the case of many potent inhibitors, where the v_s dependence on $[I]$ approaches zero more rapidly than predicted by eq 3. The corresponding $K_i(ss)$ are within parentheses in table II. These deviations are not cured, for instance, with the equation for tight binding inhibitors [25] and must be considered as yet further evidence of non-reversibility.

Inhibition of elastolytic HLE activity

The tetrapeptide used for measuring the kinetic parameters of HLE is not a true natural substrate. Kinetic arguments have been raised on the limits of slow

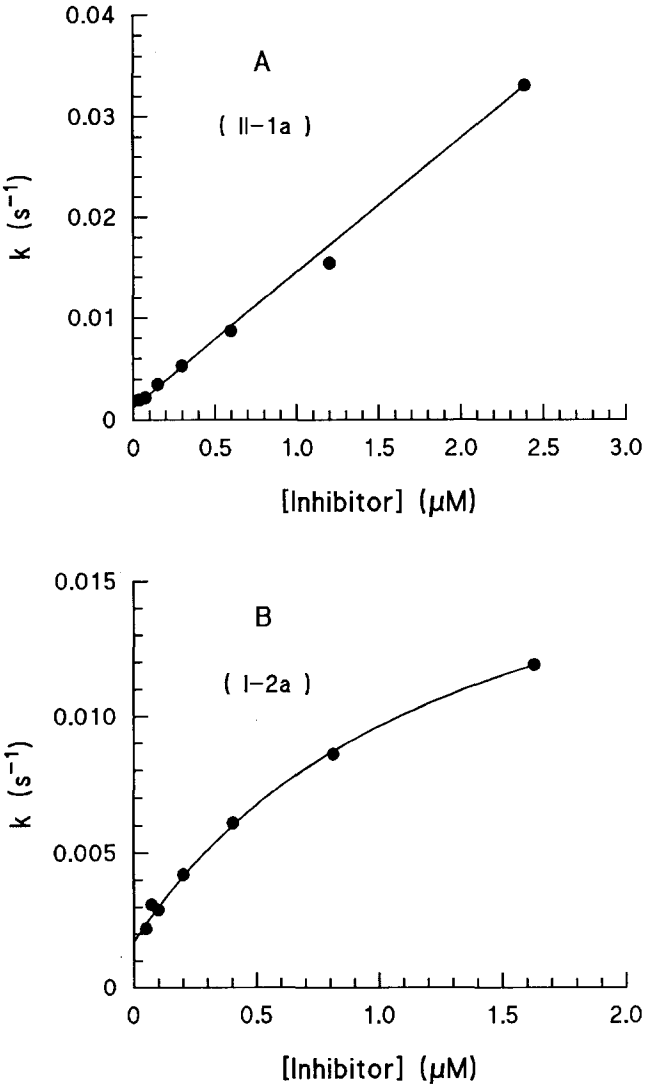


Fig 1. Analysis of kinetic data of two HLE inhibitors. Values of apparent first order rate constant, k (eq 1), are plotted *versus* the inhibitor concentration. The two panels report two experimental cases which have been analyzed with eq 2b (panel A) and 2a (panel B). The tested compounds and the best-fitting values of inhibition parameters are: A) **II–1a**, k_{on} = 18 000 M^{-1} s^{-1}, k_{off} = 0.0012 s^{-1}; B) **I–2a**, k_2 = 0.018 s^{-1}, k_{off} = 0.0017 s^{-1}, K_i = 1.0 μM. Both experiment series were carried out at constant substrate concentration so that $[S]/K_m = 0.35$.

acting inhibitors of endopeptidases in the presence of naturally-occurring protein substrates [26]. The physiological implications of the interaction between HLE and either soluble protein substrates or insoluble elastin have also been discussed [27]. Three representative compounds (**I–7a**, **II–7a** and **II–8c**) were tested as inhibitors of HLE in the presence of insoluble elas-

tin as the substrate (fig 2). Elastolysis by HLE is a slow process and the assay must be carried out with relatively high enzyme concentration and long incubation periods in order to obtain reliable readings of released elastin peptides. A good compromise was that of using an elastase concentration of 0.5 μM and an incubation time of 2 h. Since the enzyme concentration in the elastolytic assay was 200–500 times higher than that used in the kinetic experiments (table II), higher inhibitor concentrations were also necessary. Figure 2 shows a concentration-dependent inhibition of HLE elastolytic activity and represents an average effect of: i) true enzyme inhibition; ii) reactivation of the enzyme according to scheme 6; iii) spontaneous hydrolysis of the inhibitor during the incubation period. By taking into account these factors, it can be safely assumed that elastolysis by HLE is efficiently inhibited by the tested compounds.

Discussion

A comparison of inhibitory performance *versus* chemical stability between esters and thiolesters has been our primary concern and to this aim the 7α-chloro-1,1-dioxocephem nucleus was selected as a template for the preparation of a series of esters (**I–1a** to **I–4a** in table I) and thiolesters of comparable structure (**II–1a** to **II–4a**), which were then assayed with HLE.

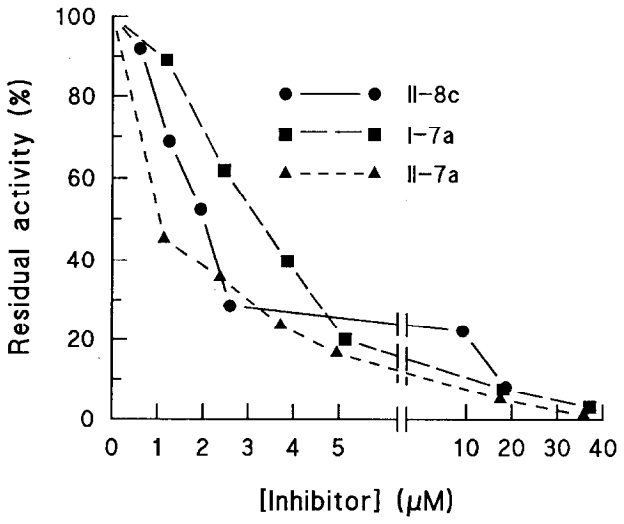


Fig 2. Inhibition of HLE elastolytic activity by three cephalosporin derivatives. The substrate was bovine neck ligament elastin incubated with 0.5 μM HLE for 2 h in the presence of various concentrations of **II–8c**, **I–7a** and **II–7a** as indicated. The percentage of residual activity refers to a control (100%) in the absence of inhibitors. Points are mean values of duplicate measurements.

This choice was motivated on the basis of the published second order rate constant for compound **I–3a** (k_2/K_i = 161 000 M^{-1} s^{-1}), which is the highest in a series of cephem esters and amides [3, 5, 6]. In spite of slightly different experimental conditions, our determination practically coincides with the above value, and thus enforces other possible comparisons. The thiolester **II–3a** is about four times as fast at inhibiting HLE, but is also hydrolysed twice as rapidly. This pattern is approximately maintained when *tert*-butyl esters with different 3'-substituents (**I–1a** to **I–4a**) are compared with the corresponding *tert*-butyl thiolester derivatives: the more electron-withdrawing substituents (S-Tet> OAc> OMe> H) correspond to the faster and less chemically stable inhibitors in the two series. Nevertheless, increments of activity up to 10-fold are recorded on passing from the ester to the thiolester series.

As a suitable leaving group at 3' (*eg* OAc or S-Tet) is a structural requirement for long lasting HLE inhibition (see above), we did not expect further possible improvements on this side. Therefore, the effect of the C-4 attached moiety was investigated in more detail by keeping the 7α-Cl and 3'-OAc as common structural features. In the ester series HLE seems to better accommodate large aliphatic and aromatic groups at this position (compare **I–3a** and **I–3b** or **3c**). However, the chemical half-life is greatly reduced when the branched carbon atom is not bound directly to the carboxyl group. This greatly limits the choice of useful substituents. In the thiolester series, on the other hand, bulky groups connected to the sulfur atom through a methylene spacer lead to both improved chemical stability and very high k_{on} values (see, for instance, **II–3c** and **II–3j**). These cysteinyl derivatives, in particular, are very attractive in view of their expected biocompatibility and possible dual-action implications. In order to preserve activity and stability, masking of the carboxyl and amino function of cysteine appears necessary (compare **II–3i** and **II–3k** with **II–3c** and **II–3j**).

To further increase stability while keeping activity at useful levels, 7-methoxy derivatives were examined. The 7-methoxy esters are in fact more stable than 7-chloro esters, at the expense however of about 10-fold decreased k_{on} values (compare **I–6a** and **I–1a**, **I–7a** and **I–3a**, **I–8a** and **I–4a**). The 7-methoxy thiolesters perform better in this comparison, thanks to a higher stability improvement and a reduced loss in k_{on} values. The result is a neat advantage of thiolesters *vs* esters in the 7-methoxy series: compounds **II–7a**, **II–8a**, **II–8c** have k_{on} in the 10^5 M^{-1} s^{-1} range as the best 7-chloro esters and chemical half lives of 6–8 h.

Although thiolesters are chemically prone to nucleophilic attack *per se*, the higher reactivity of the β-lactam moiety makes their hydrolytic lability irrele-

vant. This was experimentally checked on compounds **II–3e** and **II–3j**, whose hydrolysis in pH 7.4 phosphate buffer did not yield detectable amounts (HPLC) of the parent acid at any time. Nonetheless, thiolester hydrolysis might compete with hydrolysis of the β-lactam in the most stable compounds, *eg* **II–6a**, and in any case is bound to occur sooner or later after β-lactam cleavage. For this reason, thiolesters releasing biocompatible mercaptans (the cysteinyl derivatives **II–3i**, **II–3j**, **II–3k**, **II–6c**, **II–8c**) were included in our programme; compound **II–8c**, in particular, was successfully tested in the inhibition of HLE in the presence of insoluble elastin as the substrate. Actually, *N*-acetyl-L-cysteine (NAC) and related compounds are well-known mucolytic agents and protect the pulmonary tissue against damage mediated by neutrophil-generated oxidants [28]. The progressive release of NAC benzhydryl ester after hydrolytic cleavage (pH 7.4) of **II–3j** was confirmed, on a qualitative basis, by HPLC. Since the timespan of HLE inhibition by cephalosporin sulphones extends to a period of several hours [6], at the molecular level of the acylenzyme intermediate chances exist for an entropically favoured attack by His-57 (or other nucleophilic residues around the active site) on the reactive thiolester carbonyl. While an increased efficiency for irreversible inhibition was apparent for the thiolesters of the 7-methoxy series, as opposed to the corresponding esters, further work is needed to challenge this possibility. We are currently trying to elucidate the chemical structure of products arising from cephem thiolesters following inhibition of HLE. At the same time, the synthesis and evaluation of other modified cephalosporins incorporating an electrophilic center at the C-4 position is in progress [29, 30].

Finally, a comparative test of HLE inhibition in the presence of a natural substrate (insoluble elastin) was performed with an ester and a thiolester of otherwise identical structure (**I–7a** and **II–7a**). A second thiolester (**II–8c**) was included in this assay, in view of its biological relevance. While at saturating concentrations the inhibition was complete for all the three compounds, the thiolester **II–7a** was superior to the corresponding ester **I–7a** in the lower inhibitor concentration range. This behaviour reflects both a higher kinetic efficiency of **II–7a** *versus* **I–7a** (k_{on} = $1.4 \cdot 10^5$ and $1.1 \cdot 10^4$ M^{-1} s^{-1}, respectively) and a higher hydrolytic stability ($t_{1/2}$ = 7.0 and 3.2 h, respectively). The efficiency of **II–8c** as an inhibitor of elastolysis was roughly intermediate between that of **I–7a** and **II–7a**.

In conclusion, thiolesters of cephem sulphones behave as efficient bioisosteres of the corresponding esters in the inhibition of HLE and PPE. Apparently, they enhance the hydrolytic stability of the β-lactam moiety while preserving its full ability to acylate the Ser-195 hydroxyl of the enzyme catalytic triad. Benefits may be expected in a prospective therapeutic utilization, with further possible advantages arising from the increased stability of thiolesters *vs* esters towards serum and tissue aspecific esterases [31].

Experimental protocols

Chemistry

General procedures

Purifications by flash-chromatography were performed on columns packed with silica gel Merck 60 (230–400 mesh) and elution was carried out with cyclohexane-ethyl acetate mixtures unless otherwise stated. Melting points were determined on a Büchi apparatus and are uncorrected; all the tested compounds melted with decomposition. The ^{1}H-NMR spectra (δ ppm, tetramethylsilane as internal standard) were obtained on Varian EM-390 (90 MHz) or VXR-200 (200 MHz) spectrometers; the IR spectra were obtained on a Perkin–Elmer 1420 spectrophotometer. Analytical results for compounds followed by elemental symbols were ± 0.4% of calculated values and were determined on a Carlo–Erba NA-1005 analyzer. FD mass spectra were recorded on a Varian Mat 311/A instrument equipped with a combined EI/FI/FD ion source using benzonitrile activated emitters. The following preparative protocols were routinely used:

Protocol A. General esterification method of cephem-4-carboxylic acids. A solution of the carboxylic acid (1 mmol) in dry tetrahydrofuran (10 ml) was cooled to 0°C under nitrogen and sequentially treated with oxalyl chloride (0.174 ml, 2 mmol) and a catalytic amount (0.02 ml) of dimethylformamide. After stirring for 2 h at 0°C, the solvent was removed under vacuum (bath temperature ≤ 25°C). The residue was taken up with dry tetrahydrofuran (10 ml) and cooled to − 20°C, at which temperature the appropriate alcohol (1.2 mmol) and triethylamine (0.167 ml, 1.2 mmol) were added. The solution was stirred for 15 min at − 20°C and for 1.5 h at 0°C. The reaction mixture was diluted with dichloromethane (50 ml) and washed sequentially with 2% aqueous HCl, water, 4% aqueous $NaHCO_3$ and brine. The organic layer was dried over anhydrous sodium sulphate and rotoevaporated. The residue was purified by flash-chromatography.

Protocol B. General method for the preparation of thiolesters from cephem-4-carboxylic acids. The carboxylic acid (1 mmol) was dissolved in dichloromethane (4 ml) and sequentially treated at − 20°C with pyridine (0.6 ml), the appropriate mercaptan (1.2 mmol), and freshly prepared polyphosphate ester (EPP, 4 ml). The reaction mixture was left to stand at 0°C for 5 h and then poured under vigorous stirring into a mixture of ethyl acetate and aqueous sodium bicarbonate. The organic phase was dried over anhydrous sodium sulphate and concentrated *in vacuo*. The residue was purified by flash-chromatography.

Protocol C. General procedure for the allylic bromination of 3-methylcephems. A solution of the 3-methylcephem (1 mmol) in tetrachloromethane (20 ml) containing *N*-bromosuccinimide (NBS, 196 mg, 1.1 mmol) and a catalytic amount of azo-bis-isobutyronitrile (AIBN, 5 mg) was heated under nitrogen at reflux temperature for 5 h. The mixture was diluted with dichloromethane (30 ml), washed with 4% aqueous

$NaHSO_3$, dried over anhydrous sodium sulphate and rotoevaporated. The 3-bromomethylcephem products were purified, if desired, by flash-chromatography.

Protocol D. Deprotection of *tert*-butyl cephem-4-carboxylates. A solution of the *tert*-butyl ester (1 mmol) in a mixture of dichloromethane, trifluoroacetic acid, and anisole (1:1:0.5, 10 ml) was left to stand at room temperature for 30 min. The solution was concentrated to a small volume, taken up with toluene and rotoevaporated to dryness, affording the crude carboxylic acid product, which was further processed without purification.

Preparation of tert-butyl 7α-chloro-3-methyl-3-cephem-4-carboxylate 1,1-dioxide ***I–1a***

A mixture of 7-ADCA (21.4 g, 0.1 mol) in acetone (600 ml) and water (250 ml) was chilled to – 10°C. To this solution, 37% HCl (100 ml) was added slowly under stirring, while keeping the temperature between –10°C and – 5°C, followed by a solution of sodium nitrite (15.2 g, 0.22 mol) in water (50 ml). The resulting mixture was stirred for 1 h, during which time the temperature was allowed to rise to + 10°C, and then poured into dichloromethane (300 ml) and water (250 ml). The aqueous phase was extracted with another portion of dichloromethane (200 ml), and the combined organic extracts were washed with water (250 ml), dried over anhydrous sodium sulphate and concentrated *in vacuo*. The obtained brownish syrup (*ca* 20 g) was dissolved in 50% aqueous methanol (800 ml) and treated with potassium peroxymonosulphate (Oxone, 100 g). The mixture was heated at 55°C for 100 min under vigorous stirring, thence cooled to room temperature and reduced to about one half of its initial volume by rotoevaporation. After partitioning between ethyl acetate and water, the organic layer was rinsed twice with brine, dried over anhydrous sodium sulphate and concentrated under reduced pressure. The residue was treated with diisopropyl ether, stirred for 30 min and the product, consisting of crude 7α-chloro-3-methyl-3-cephem-4-carboxylic acid 1,1-dioxide **12**, was collected by filtration as a white powder (15 g). ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.54 (9H, s); 2.07 (3H, s); 3.66 (1H, d, J = 18.0 Hz); 3.83 (1H, br d, J = 18.0 Hz); 4.71 (1H, br d); 5.25 (1H, d, J = 1.6 Hz). IR (KBr): 3500–2500, 1800, 1730, 1695 cm^{-1}.

Esterification of the acid **12** with *tert*-butyl alcohol was performed according to *Protocol A*. The product **I–1a** was obtained as a white powder (10.5 g, 33%), mp: 171–175°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.52 (9H, s); 2.05 (3H, s); 3.65 (1H, d, J = 18.2 Hz); 3.86 (1H, br d, J = 18.2 Hz); 4.70 (1H, br s); 5.24 (1H, d, J = 1.5 Hz). IR (KBr): 1783, 1784 cm^{-1}. Anal $C_{12}H_{16}ClNO_5S$ (C, H, N).

Preparation of tert-butyl 7α-chloro-3-methoxymethyl-3-cephem-4-carboxylate 1,1-dioxide ***I–2a***

A solution of 3-acetoxymethyl-7α-chloro-3-cephem-4-carboxylic acid **17** [11] (9.92 g, 34 mmol) in methanol (110 ml) and water (50 ml) was treated with a solution of sodium bicarbonate (2.7 g) in water (120 ml). Calcium chloride (187 g) was added and the mixture was heated at 70°C for 75 min. After cooling to 10°C, the mixture was made acidic with 37% HCl (7 ml) and extracted with ethyl acetate (2 x 350 ml). The extracts were washed with brine, dried over anhydrous sodium sulphate and concentrated *in vacuo*. The residue was taken up with ethyl ether (150 ml) and stirred for 30 min. The insoluble material, mainly 7α-chlorodeacetylcephalosporanic acid lactone, was discarded. The solution was concentrated to afford crude 7α-chloro-3-methoxymethyl-3-cephem-4-carboxylic acid as a yellowish oil (4 g). This material was dissolved in 50% aqueous methanol (140 ml) and treated with Oxone (16 g) at 60°C for 90 min. After concentration *in vacuo*, the reaction mixture was partitioned between ethyl acetate and water. Evaporation of the solvent from the organic extract left crude 7α-chloro-3-methoxymethyl-3-cephem-4-carboxylic acid 1,1-dioxide **18** as a whitish solid (3.5 g). IR (KBr): 1798, 1716 cm^{-1}.

Esterification of the acid **18** was performed according to the procedure reported in *Protocol A*. The product **I–2a** was isolated as a white powder (1.7 g, 14% yield), mp: 127–130°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.54 (9H, s); 3.33 (3H, s); 3.86 (1H, d, J = 18.3 Hz); 4.02 (1H, br d, J = 18.3 Hz); 4.26 (2H, s); 4.76 (1H, dd, J = 1.5 and 1.7 Hz); 5.28 (1H, d, J = 1.7 Hz). IR (KBr): 1790, 1720 cm^{-1}. Anal $C_{13}H_{18}ClNO_6S$ (C, H, N).

Preparation of tert-butyl 3-acetoxymethyl-7α-chloro-3-cephem-4-carboxylate 1,1-dioxide ***I–3a***

Following the procedure reported in *Protocol A*, 3-acetoxymethyl-7α-chloro-3-cephem-4-carboxylic acid 1,1-dioxide **15** [11] was esterified with *tert*-butyl alcohol to afford **I–3a** as a white powder (63% yield). Spectral data were consistent with those reported in the literature [3].

Preparation of benzyl 3-acetoxymethyl-7α-chloro-3-cephem-4-carboxylate 1,1-dioxide ***I–3b***

Following the procedure reported in *Protocol A*, 3-acetoxymethyl-7α-chloro-3-cephem-4-carboxylic acid 1,1-dioxide **15** was esterified with benzyl alcohol to afford **I–3b** as a colorless oil (71% yield). ^{1}H-NMR ($CDCl_3$, 90 MHz): 2.05 (3H, s); 3.96 (2H, ABq, J = 18 Hz); 4.88 (2H, ABq, J = 14 Hz); 4.89 (1H, br s); 5.23 (1H, d, J = 2 Hz); 7.4 (5H, s). IR ($CHCl_3$): 1820, 1740 cm^{-1}. FD-MS: 413 (M^+).

Preparation of (S)-2-benzhydryloxycarbonyl-2-tert-butoxycarbonylaminoethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-carboxylate 1,1-dioxide ***I–3c***

Obtained from acid **15** and *N*-Boc-L-serine benzhydryl ester according to *Protocol A* as a foam (41% yield). ^{1}H-NMR ($CDCl_3$, 400 MHz): 3.74 (1H, d, J = 18.0 Hz); 3.96 (1H, br d, J = 18.0 Hz); 4.64 (1H, dd, J = 3.5 and 11.0 Hz); 4.68 (1H, m); 4.71 (1H, dd, J = 1.5 and 1.7 Hz); 4.78 (1H, dd, J = 3.5 and 11.0 Hz); 4.53 and 4.93 (2H, two d, J = 14.4 Hz); 5.28 (1H, d, J = 1.7 Hz); 5.65 (1H, d, J = 8.0 Hz); 6.86 (1H, s); 7.2–7.3 (10H, m). IR (KBr): 3400, 1815, 1750, 1720 cm^{-1}. FD-MS: 677 *m/z* (M^+).

Preparation of tert-butyl 7α-chloro-3-(1-methyl-1,2,3,4-tetrazol-5-yl)-thiomethyl-3-cephem-4-carboxylate 1,1-dioxide ***I–4a***

A solution of the 3-methylcephem **I–1a** (5 g, 15.5 mmol) in tetrachloromethane (310 ml) was brominated and worked up as indicated in protocol C to afford *tert*-butyl 3-bromomethyl-7α-chloro-3-cephem-4-carboxylate 1,1 dioxide **13** as a white solid (3.3 g). ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.57 (9H, s); 3.82 (1H, d, J = 17.0 Hz); 4.18 (1H, br d, J = 17.0 Hz); 4.15 and 4.49 (2H, two d, J = 10.9 Hz); 4.81 (1H, br s); 5.30 (1H, J = 1.7 Hz). IR (KBr): 1805, 1720 cm^{-1}.

A portion of this product (200 mg, 0.5 mmol) was added to a solution of 5-mercapto-1-methyl-1,2,3,4-tetrazole (60 mg, 0.53 mmol) and triethylamine (0.07 ml, 0.5 mmol) in acetonitrile (15 ml). The mixture was stirred for 15 min and then partitioned between ethyl acetate and water. The organic phase was concentrated to a small volume and ethyl ether was added. The product **I–4a** was collected by filtration as a white powder (0.16 g, 41% overall yield), mp: 176–177°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.57 (9H, s); 3.93 (3H, s); 4.10 (1H, d, J = 18.0 Hz); 4.32 (1H, br d, J = 18.0 Hz); 4.10 and 4.51 (2H, two d, J = 14.1 Hz); 4.76 (1H, dd, J = 1.5 and 1.6 Hz); 5.27 (1H, d, J = 1.6 Hz). IR (KBr): 1795, 1712 cm^{-1}. Anal $C_{14}H_{18}ClN_5O_5S_2$ (C, H, N).

Preparation of tert-butyl 7α-methoxy-3-methyl-3-cephem-4-carboxylate 1,1-dioxide ***I–6a***
Obtained from acid **22** and *tert*-butyl alcohol according to *Protocol A* as a white powder (64% yield), mp: 140–142°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.54 (9H, s); 2.03 (3H, s); 3.56 (3H, s); 3.59 (1H, d, J = 17.8 Hz); 3.83 (1H, br d, J = 17.8 Hz); 4.62 (1H, br s); 5.13 (1H, d, J = 1.5 Hz). IR (KBr): 1785, 1718 cm^{-1}. Anal $C_{13}H_{19}NO_6S$ (C, H, N).

Preparation of tert-butyl 7α-methoxy-3-(1-methyl-1,2,3,4-tetrazol-5-yl)-thiomethyl-3-cephem-4-carboxylate 1,1-dioxide ***I–8a***
The 3-methylcephem **I–6a** (2 g, 6.3 mmol) was brominated according to *Protocol C*. The obtained product, *tert*-butyl 3-bromomethyl-7α-methoxy-3-cephem-4-carboxylate, was added to a solution of 5-mercapto-1-methyl-1,2,3,4-tetrazole (0.6 g) and triethylamine (0.7 ml) in acetonitrile (100 ml). The mixture was stirred for 15 min and then partitioned between ethyl acetate and water. The residue obtained by rotoevaporation of the organic extract was purified by flash-chromatography. The product **I–8a** was obtained as a white powder after trituration with ethyl ether (1.22 g, 45% yield). Spectral data were consistent with those reported in the literature [5].

Preparation of tert-butyl 7α-chloro-3-methyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–1a***
Obtained from crude acid **12** and *tert*-butyl mercaptan according to *Protocol B* as a white powder (53% yield), mp: 170–174°C. ^{1}H-NMR ($CDCl_3$, 90 MHz): 1.55 (9H, s); 2.07 (3H, s); 3.77 (2H, ABq, J = 18 Hz); 4.73 (1H, br s); 5.32 (1H, d, J = 1.5 Hz). IR (KBr): 1800, 1660 cm^{-1}. Anal $C_{12}H_{16}ClNO_4S_2$ (C, H, N).

Preparation of tert-butyl 7α-chloro-3-methoxymethyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–2a***
Obtained from crude acid **18** and *tert*-butyl mercaptan according to *Protocol B* as a white powder (39% yield), mp: 100–105°C. ^{1}H-NMR ($CDCl_3$, 90 MHz): 1.55 (9H, s); 3.35 (3H, s); 3.96 (2H, ABq, J = 16.5 Hz); 4.17 (2H, s); 4.80 (1H, br s); 5.34 (1H, d, J = 1.8 Hz). IR (KBr): 1790, 1665 cm^{-1}. Anal $C_{13}H_{18}ClNO_5S_2$ (C, H, N).

Preparation of tert-butyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3a***
Obtained from acid **15** and *tert*-butyl mercaptan according to *Protocol B* as a white powder (73% yield), mp: 179–182°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.60 (9H, s); 2.11 (3H, s); 3.92 (2H, ABq, J = 18 Hz); 4.81 (2H, ABq, J = 14 Hz), 4.85 (1H, br s); 5.37 (1H, d, J = 2 Hz). IR (KBr): 1790, 1735, 1665, 1650 cm^{-1}. Anal $C_{14}H_{18}ClNO_6S_2$ (C, H, N).

Preparation of benzyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3b***
Acid **15** and benzyl mercaptan were condensed with EPP according to *Protocol B*, and the crude product was fractionated by flash-chromatography. The first-eluted product was benzyl 3-acetoxymethyl-3-benzylthio-7α-chloro-3-cepham-4-thiolcarboxylate 1,1-dioxide **20**, obtained as a colorless oil (31% yield). ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.97 (3H, s); 3.30 and 3.91 (2H, two d, J = 15.2 Hz); 3.8–4.2 (6H, m); 4.46 (1H, s); 5.00 (1H, d, J = 1.5 Hz); 5.42 (1H, d, J = 1.5 Hz); 7.2–7.3 (10H, m). IR ($CHCl_3$): 1805, 1745, 1660 cm^{-1}. FD-MS: 553 (M^+), 489 *m/z* (M^+-SO_2).

The second-eluted product was the thiolester **II–3b**, obtained as a white powder (15% yield), mp: 158–161°C. ^{1}H-NMR ($CDCl_3$, 90 MHz): 2.07 (3H, s); 3.96 (2H, ABq, J = 18.5 Hz); 4.32 (2H, s); 4.86 (2H, ABq, J = 13.5 Hz); 4.87 (1H, br s); 5.35 (1H, d, J = 1.8 Hz); 7.37 (5H, s). IR (KBr): 1812, 1738, 1655 cm^{-1}. Anal $C_{17}H_{16}ClNO_6S_2$ (C, H, N).

Preparation of (S)-2-benzhydryloxycarbonyl-2-tert-butoxycarbonylaminoethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3c***
Obtained from acid **15** and *N*-Boc-L-cysteine benzhydryl ester according to *Protocol B* as a white powder (72% yield), mp: 95–105°C. ^{1}H-NMR (200 MHz, $CDCl_3$): 1.41 (9H, s); 2.08 (3H, s); 3.38 (1H, dd, J = 5.5 and 14.1 Hz); 3.73 (1H, d, J = 18.2 Hz); 3.86 (1H, dd, J = 4.3 and 14.1 Hz); 4.00 (1H, d, J = 18.2 Hz); 4.54 (1H, d, J = 14.0 Hz); 4.78 (1H, m); 4.79 (1H, m); 5.03 (2H, d, J = 14.0 Hz); 5.33 (1H, d, J = 1.7 Hz); 6.90 (1H, s); 7.33 (10H, s). IR (KBr): 1807, 1738, 1710 (br), 1660 (sh) cm^{-1}. Anal $C_{31}H_{33}ClN_2O_{10}S_2$ (C, H, N).

Preparation of n-butyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3d***
n-Butanethiol (0.107 ml, 1 mmol) and dicyclohexylcarbodiimide (206 mg, 1 mmol) were added, at room temperature, to a stirred solution of the acid **15** (323 mg, 1 mmol) in dichloromethane (12 ml). After 10 minutes the solvent was removed *in vacuo* and the residue was fractionated by flash-chromathography. The first-eluted product was the 4-spiro-2-cephem **19** [11] (332 mg, 65% yield). The product **II–3d** was eluted second and obtained as a white solid (90 mg, 23% yield), mp: 130–133°C. ^{1}H-NMR ($CDCl_3$, 90 MHz): 0.93 (3H, t, J = 6.2 Hz); 1.1–2.0 (4H, m); 2.11 (3H, s); 3.08 (2H, d, J = 6.3 Hz); 3.92 (2H, ABq, J = 17.5 Hz); 4.83 (1H, br s); 4.84 (2H, ABq, J = 14.5 Hz); 5.34 (1H, d, J = 1.8 Hz). IR (KBr): 1812, 1735, 1650 cm^{-1}. Anal $C_{14}H_{18}ClNO_6S_2$ (C, H, N).

Preparation of isopropyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3e***
Obtained from acid **15** and isopropyl mercaptan according to *Protocol B* as a white powder (79% yield), mp: 115–118°C. ^{1}H-NMR ($CDCl_3$, 90 MHz): 1.41 (6H, s); 2.05 (3H, s); 3.28 (2H, t, J = 6.0 Hz); 3.88 (2H, t, J = 6.0 Hz); 3.95 (2H, ABq, J = 18.0 Hz); 4.83 (2H, ABq, J = 14.2 Hz); 4.86 (1H, br s); 5.38 (1H, d, J = 1.7 Hz); 7.3–7.6 (10H, m). IR (KBr): 1810, 1740, 1660 cm^{-1}. Anal $C_{13}H_{16}ClNO_6S_2$ (C, H, N).

Preparation of 2-phenylethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3f***
Acid **15** and 2-phenylethyl mercaptan were condensed according to *Protocol B*. Following silica gel chromatography, 2-phenylethyl 3-acetoxymethyl-7α-chloro-3-(2-phenylethyl)-thio-3-cepham-4-thiolcarboxylate 1,1-dioxide **21** was isolated as a colorless oil (41% yield). ^{1}H-NMR ($CDCl_3$, 90 MHz): 2.02 (3H, s); 2.7–3.4 (8H, m); 3.52 (2H, ABq, J = 15 Hz); 4.03 (2H, ABq, J = 12 Hz); 4.43 (1H, s); 4.95 (1H, d, J = 1.7 Hz); 5.41 (1H, d, J = 1.7 Hz); 7.28 (10H, s). IR ($CHCl_3$): 1810, 1755, 1670 cm^{-1}. FD-MS 581 (M^+). A solution of the cepham **21** (128 mg, 0.22 mmol) in dichloromethane (20 ml) was cooled to – 20°C and treated with 70% 3-chloroperoxybenzoic acid (70 mg, 0.28 mmol). The mixture was stirred for 15 min at – 20°C, and then sequentially washed with saturated aqueous $NaHSO_3$, water, 4% aqueous $NaHCO_3$ and brine. Removal of the solvent and chromatography of the residue allowed the isolation of the unreacted starting material (45 mg) and the less polar product **II–3f** as a white powder (60 mg, 93% yield based on converted **11**, 25% yield from **15**), mp: 133–135°C. ^{1}H-NMR ($CDCl_3$, 90 MHz): 2.10 (3H, s); 3.0 and 3.30 (4H, each d, J = 7 Hz); 3.75 and 4.05 (2H, each d, J = 18 Hz); 4.65 and 4.97 (2H, each d, J = 14 Hz); 4.81 (1H, br s); 5.35 (1H, d, J = 1.8 Hz). IR (KBr): 1815, 1730, 1640 cm^{-1}. Anal $C_{18}H_{18}ClNO_6S_2$ (C, H, N).

Preparation of benzhydryloxycarbonylmethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3g***
Obtained from acid **15** and benzhydryl mercaptoacetate according to *Protocol B* as an amorphous solid (75 yield). ^{1}H-NMR ($CDCl_3$, 90 MHz): 2.07 (3H, s); 3.93 (2H, ABq, J = 18 Hz); 3.97 (2H, s); 4.80 (2H, ABq, J = 14.5 Hz); 4.80 (1H, br s); 5.34 (1H, d, J = 1.7 Hz); 6.93 (1H, s); 7.39 (10H, s). IR (KBr): 1808, 1740, 1665 cm^{-1}. Anal $C_{25}H_{22}ClNO_8S_2$ (C, H, N).

Preparation of carboxymethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3h***
A solution of the benzhydryl ester **II–3g** (180 mg, 0.32 mmol) in dichloromethane (20 ml), anisole (2 ml) and trifluoroacetic acid (2 ml) was left to stand at 0°C for 2.5 h. The solvent was removed by rotoevaporation and the residue was purified by flash-chromatography. The product **II–3g** was isolated as a white powder after trituration in ethyl ether (105 mg, 83% yield), mp: 140–143°C. ^{1}H-NMR (DMSO–d_6, 200 MHz): 2.03 (3H, s); 3.88 (2H, s); 4.42 (2H, ABq, J = 18 Hz); 4.70 (2H, ABq, J = 13.5 Hz); 5.72 (1H, d, J = 1.5 Hz); 5.96 (1H, d, J = 1.5 Hz). IR (KBr): 1815–1810, 1740–1720, 1655 cm^{-1}. Anal $C_{12}H_{12}ClNO_8S_2$ (C, H, N).

Preparation of (S)-2-amino-2-carboxyethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3i***
A solution of the benzhydryl ester **II–3c** (118 mg, 0.17 mmol) in trifluoroacetic acid, dichloromethane and anisole (1:8:1, 15 ml) was left at room temperature for 5 h, and thence evaporated to dryness. The residue was triturated in a mixture of dichloromethane and ethyl ether (1:1). The product **II–3i** was collected by centrifugation as a white powder (42 mg, 53% yield), mp: 165–170°C. ^{1}H-NMR (DMSO–D_2O, 200 MHz): 2.01 (3H, s); 3.35 (1H, dd, J = 6.6 and 13.8 Hz); 3.52 (1H, dd, J = 13.8 and 5.8 Hz); 3.66 (2H, obscured); 3.74 (1H, m); 4.70 (2H, ABq, J = 13.6 Hz); 5.66 (1H, d, J = 1.6 Hz); 5.92 (1H, d, J = 1.6 Hz). IR (KBr): 1808, 1742, 1670, 1620 cm^{-1}. Anal $C_{13}H_{15}ClN_2O_8S_2$ + 0.25 TFA (C, H, N).

Preparation of (S)-2-acetamido-2-benzhydryloxycarbonylethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3j***
Obtained from acid **15** and *N*-acetyl-L-cysteine benzhydryl ester according to *Protocol B* as a foam (39%). ^{1}H NMR ($CDCl_3$, 200 MHz): 1.96 (3H, s); 2.07 (3H, s); 3.47 (1H, dd, J = 5.1 and 14.2 Hz); 3.74 (1H, d, J = 17.7 Hz); 3.80 (1H, dd, J = 4.3 and 14.2 Hz); 3.99 (1H, br d, J = 17.7 Hz); 4.81 (1H, dd, J = 1.5 and 1.8 Hz); 4.49 and 4.45 (2H, two d, J = 13.8 Hz); 5.09 (1H, ddd, J = 4.3, 5.1 and 8.1 Hz); 5.33 (1H, d, J = 1.8 Hz); 6.60 (1H, d, J = 8.1 Hz); 6.87 (1H, s); 7.2–7.4 (10H, m). IR ($CHCl_3$): 1820, 1745, 1670 cm^{-1}. FD-MS: 635 (MH^+), 634 *m/z* (M^+).

Preparation of (S)-2-acetamido-2-carboxyethyl 3-acetoxymethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–3k***
A solution of the benzhydryl ester **II–3j** (146 mg, 0.23 mmol) in dichloromethane (10 ml), trifluoroacetic acid (3 ml) and anisole (0.5 ml) was left to stand at room temperature for 1 h, and then concentrated to dryness. After trituration in ethyl ether, the product **II–3k** was collected by filtration (91 mg, 85% yield), mp: 170–175°C. ^{1}H-NMR (DMSO–d_6, 200 MHz): 1.80 (3H, s); 2.01 (3H, s); 3.20 (1H); 3.52 (1H, dd, J = 5.0 and 13.8 Hz); 4.41 (1H, d, J = 18.2 Hz); 4.44 (1H, br d, J = 18.2 Hz); 4.44 (1H, m); 4.58 and 4.75 (2H, two d, J = 13.5 Hz); 5.68 (1H, d, J = 1.2 Hz); 5.90 (1H, br s); 8.15 (1H, d, J = 8.2 Hz). IR ($CHCl_3$): 3380, 1810, 1785, 1745, 1675, 1645 (sh) cm^{-1}. Anal $C_{15}H_{17}ClN_2O_9S_2$ (C, H, N).

Preparation of tert-butyl 7α-chloro-3-(1-methyl-1,2,3,4-tetrazol-5-yl)-thiomethyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–4a***
From the 3-acetoxymethylcephem **15**: A solution of **15** (400 mg, 1.24 mmol) and 5-mercapto-1-methyl-1,2,3,4-tetrazole (200 mg, 1.72 mmol) in acetonitrile (15 ml) was treated with boron trifluoride etherate (0.5 ml) and heated at 50°C for 6 h. Evaporation of the solvent and trituration of the residue in ethyl ether afforded crude 7α-chloro-3-(1-methyl-1,2,3,4-tetrazol-5-yl)-thiomethyl-3-cephem-4-carboxylic acid **16** (430 mg). IR (KBr): 1810, 1720 cm^{-1}.

The acid **16** and *tert*-butyl mercaptan were condensed as described in *Protocol B*. The product **II–4a** was obtained as a white amorphous solid (118 mg, 21% yield). ^{1}H-NMR ($CDCl_3$, 90 MHz): 1.55 (9H, s); 3.98 (3H, s); 4.1–4.5 (4H, two ABq); 4.87 (1H, br s); 5.35 (1H, d, J = 1.7 Hz). IR (KBr): 1810, 1655 cm^{-1}. Anal $C_{14}H_{18}ClN_5O_4S_3$ (C, H, N).

From the *tert*-butyl ester **I–4a**: Compound **I–4a** (218 mg, 0.5 mmol) was converted into the corresponding acid **16** (195 mg) under the conditions reported in *Protocol D*. Without purification, this intermediate was condensed with *tert*-butyl mercaptan according to *Protocol B*. The product **II–4a** described above was obtained (163 mg, 72% yield).

Preparation of tert-butyl 3-(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-1,2,4-triazin-3-yl)thiomethyl-7α-chloro-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–5a***
A solution of the 3-methylcephem thioester **II–1a** (900 mg, 2.66 mmol) in anhydrous 1,2-dichloroethane (40 ml) was treated with *N*-bromosuccinimide (473 mg, 2.66 mmol) and irradiated at – 20°C under nitrogen in a quartz vessel with a Hanovia 125 W Hg lamp for 2 h. Following evaporation of the solvent, the residue was fractionated by flash-chromatography. After elution of the unreacted starting material (350 mg), the less polar *tert*-butyl 7α-chloro-3-bromomethyl-3-cephem-4-thiolcarboxylate 1,1-dioxide **14** was isolated as a yellowish solid (80 mg, 12% yield based on converted **II–1a**); ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.57 (9H, s); 3.77 (1H, d, J = 17.8 Hz); 4.16 (1H, br d, J = 17.8 Hz); 4.07 and 4.29 (2H, two d, J = 11.1 Hz); 4.82 (1H, dd, J = 1.6 and 1.9 Hz); 5.33 (1H, d, J = 1.9 Hz). FD-MS: 415 *m/z* (M^+).

To a portion of this product (42 mg, 0.10 mmol) dissolved in acetonitrile (5 ml), 6-benzhydryloxy-2,5-dihydro-3-mercapto-5-oxo-1,2,4-triazine (39 mg, 0.12 mmol) and triethylamine (0.017 ml, 0.12 mmol) were added sequentially. The mixture was stirred for 30 min and then partitioned between water and ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and rotoevaporated to leave a residue which was purified by flash-chromatography. Without characterization, the isolated product was left to stand in a mixture of dichloromethane, trifluoroacetic acid and anisole (3:1:0.2, 4 ml) for 10 min. Evaporation under high vacuum left a residue, which was dissolved in a small amount of dichloromethane and precipitated with isopropyl ether to afford the product **II–5a** as a white powder (10 mg, 21% yield); mp: 126–127°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.55 (9H, s); 3.73 (3H, s); 3.93 and 4.20 (2H, two d, J = 17.8 Hz); 4.00 and 4.35 (2H, two d, J = 13.8 Hz); 4.75 (1H, br s); 5.28 (1H, d, J = 1.5 Hz). IR ($CHCl_3$): 1810, 1710 (sh), 1660 cm^{-1}. Anal $C_{16}H_{19}ClN_4O_6S_3$ (C, H, N).

Preparation of tert-butyl 7α-methoxy-3-methyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–6a***
Obtained from acid **22** and *tert*-butyl mercaptan according to protocol B as a white powder (73% yield), mp: 158–160°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.52 (9H, s); 2.00 (3H, s); 3.55 (1H, d, J = 17.1 Hz); 3.57 (3H, s); 3.58 (1H, br d, J = 17.1 Hz);

3.78 (1H, br d, J = 17.1 Hz); 4.59 (1H, br s); 5.12 (1H, d, J = 1.6 Hz). IR (KBr): 1780, 1660 cm^{-1}. Anal $C_{13}H_{19}NO_5S_2$ (C, H, N).

Preparation of (S)-2-benzhydryloxycarbonyl-2-tert-butoxycarbonylaminoethyl 7α-methoxy-3-methyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–6c***

Obtained from acid **22** and *N*-Boc-L-cysteine benzhydryl ester according to *Protocol B* as a foam (75% yield). ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.41 (9H, s); 2.00 (3H, s); 3.35 (1H, dd, J = 6.2 and 14.2 Hz); 3.56 (3H, s); 3.60 (1H, d, J = 17.8 Hz); 3.74 (1H, dd, J = 4.5 and 14.2 Hz); 3.80 (1H, br d, J = 17.8 Hz); 4.61 (1H, br s); 4.70 (1H, m); 5.15 (1H, d, J = 1.6 Hz); 5.35 (1H, d, J = 8.0 Hz); 6.88 (1H, s); 7.2–7.3 (10H, m). IR ($CHCl_3$): 1805, 1740, 1705, 1680 (sh) cm^{-1}. FD-MS: 630 *m/z* (M^+).

Preparation of tert-butyl 3-acetoxymethyl-7α-methoxy-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–7a***

The *tert*-butyl ester **I–7a** (375 mg, 1 mmol) was hydrolyzed as indicated in *Protocol D*, affording crude 3-acetoxymethyl-7α-methoxy-3-cephem-4-carboxylic acid 1,1-dioxide as an amorphous solid. ^{1}H-NMR (DMSO–d_6, 200 MHz): 2.02 (3H, s); 3.44 (3H, s); 4.23 (2H, br s); 4.93 and 4.56 (2H, two d, J = 13.0 Hz); 5.21 (1H, d, J = 1.5 Hz); 5.48 (1H, br s). IR ($CHCl_3$): 3600–2400, 1808, 1745 cm^{-1}.

This intermediate was condensed with *tert*-butyl mercaptan according to *Protocol A*. The product **II–7a** was obtained as a colourless oil that solidified on standing (216 mg, 53% yield), mp: 108–109°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.53 (9H, s); 2.08 (3H, s); 3.56 (3H, s); 3.62 (1H, d, J = 18.0 Hz); 3.96 (1H, br d, J = 18.0 Hz); 4.67 (1H, dd, J = 1.5 and 1.7 Hz); 4.61 and 4.87 (2H, two d, J = 13.6 Hz); 5.16 (1H, d, J = 1.7 Hz). IR (KBr): 1805, 1755, 1655, 1635 cm^{-1}. Anal $C_{15}H_{21}NO_7S_2$ (C, H, N).

Preparation of tert-butyl 7α-methoxy-3-(1-methyl-1,2,3,4-tetrazol-5-yl)-thiomethyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–8a***

Hydrolysis of the *tert*-butyl ester **I–8a** (302 mg, 0.7 mmol) was performed as indicated in *Protocol D*. The residue was triturated in ethyl ether and filtered to give 7α-methoxy-3-(1-methyl-1,2,3,4-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid 1,1-dioxide (crude, *ca* 250 mg), which was condensed with *tert*-butyl mercaptan according to *Protocol B*. The product **II–8a** was obtained as an amorphous solid (135 mg, 43% yield). ^{1}H-NMR ($CDCl_3$, 90 MHz): 1.55 (9H, s); 3.58 (3H, s); 3.88 (1H, d, J = 17.5 Hz); 3.93 (3H, s); 4.04 and 4.38 (2H, each d, J = 14 Hz); 4.27 (1H, dd, J = 17.5 and 1.1 Hz); 4.63 (1H, dd, J = 1.7 and 1.1 Hz); 5.13 (1H, d, J = 1.7 Hz). IR ($CHCl_3$): 1800, 1650 cm^{-1}. Anal $C_{15}H_{21}ClN_5O_5S_3$ (C, H, N).

Preparation of (S)-2-benzhydryloxycarbonyl-2-(tert-butoxycarbonylamino)ethyl 7α-methoxy-3-(1-methyl-1,2,3,4-tetrazol-5-yl)thiomethyl-3-cephem-4-thiolcarboxylate 1,1-dioxide ***II–8c***

Hydrolysis of the *tert*-butyl ester **I–8a** (302 mg, 0.7 mmol) was carried out as reported in *Protocol D*. The resulting crude 7α-methoxy-3-(1-methyl-1,2,3,4-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid 1,1-dioxide was condensed with *N*-Boc-L-cysteine benzhydryl ester according to *Protocol B*. The product **II–8c** was isolated as a foam (174 mg, 54% yield). ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.39 (9H, s); 3.36 (1H, dd, J = 6.8 and 14.1 Hz); 3.87 (1H, dd, J = 4.1 and 14.1 Hz); 3.90 (1H, d, J = 17.2 Hz); 3.94 (1H, d, J = 14.5 Hz); 4.23 (1H, br d, J = 17.2 Hz); 4.53 (1H, br); 4.59 (1H, d, J = 14.5 Hz); 4.7 (1H, m); 5.13 (1H, d, J = 1.6 Hz); 5.75 (1H, d, J = 8.1 Hz, exch D_2O); 6.90 (1H, s); 7.23–7.35 (10H, m). IR ($CHCl_3$): 1810, 1745 (sh), 1715, 1675 cm^{-1}. FD-MS: 744 *m/z* (M^+).

Hydrolytic stability of the inhibitors

A Hewlett–Packard 1090 LUSI chromatograph equipped with temperature controlled autosampler was used to investigate the hydrolytic stability of the cephem sulphones. The compounds were dissolved in 0.05 M pH 7.4 phosphate buffer, containing 5% dimethylsulphoxide as solubilizing vehicle, to a final concentration of 0.2 mg/ml. Time course of degradation at 37°C was followed by resolving the degradation products from the peak of the parent compound with a 5 μm Partisphere C18 cartridge (Whatman, 110 x 4.7 mm), eluting with an appropriate gradient of pH 2.5 phosphate buffer and acetonitrile. Chemical half-lives (hours) were determined from first-order kinetic analysis of decrease with time of area counts corresponding to the peak of the tested compounds. Results are presented in table II.

Enzymology

Enzymes and substrates

Porcine pancreatic elastase, PPE (EC 3.4.21.36), was purchased from Sigma; human leukocyte elastase, HLE (EC 3.4.21.37) was a Calbiochem product; the peptide substrates were either from Sigma or from Bachem (Bubendorf, Switzerland); bovine elastin was a Sigma product. HLE was dissolved at a concentration of 1–2 mg/ml in 5 mM acetate buffer pH 5, supplemented with 0.145 M NaCl and 0.01% Triton X-100, then aliquoted in plastic tubes and stored frozen at – 20°C. The active site concentration of the enzyme was determined by spectrophotometric monitoring of residual activity after reaction with increasing amounts of the irreversible inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl (Sigma).

Inhibition of PPE

Inhibition of the amidolytic PPE activity was investigated spectro-photometrically with the chromogenic substrate MeO-Suc-Ala-Ala-Pro-Ala-NH-Ph-pNO_2 [32], by monitoring the release of *p*-nitroaniline at 410 nm, in phosphate buffer 0.05 M, pH 7.4, 2.5% acetonitrile, 30°C. Results are reported as IC_{50} (table II), which are empirically defined as inhibitor concentrations (μg/ml) effective for 50% reduction of enzyme activity 6 min after mixing. These IC_{50} values cannot be used as absolute parameters such as k_{on} and K_i since they depend on the particular experimental conditions used, most notably on the substrate concentration and on the incubation time after reagent mixing. Thus they must be considered as a practical tool for comparing the relative efficiency of the inhibitors within table II, not with literature data obtained under different conditions.

Inhibition of the amidolytic activity of HLE

HLE inhibition kinetics were investigated with a High Tech SF-51 stopped-flow instrument in the fluorescence detection mode, upon monitoring the release of 7-amino-4-methyl-coumarin from the fluorogenic substrate MeO-Suc-Ala-Ala-Pro-Val-7-(4-methyl)coumarylamide [33] at a temperature of 37°C. Typically, one syringe was filled with an enzyme solution in acetate buffer, while substrate and inhibitor in phosphate buffer (55 mM, pH 7.6) were in the other syringe. The final pH, as obtained after mixing equal amounts of the two solutions, was 7.4. Both solutions contained 1% dimethylsulphoxide and 1% acetonitrile, as solubilizer of substrate and

inhibitor respectively, and 0.01% Triton X-100. The active enzyme concentration ranged from 1 to 3 nM, while substrate and inhibitor concentrations varied as required for a proper determination of kinetic parameters. The Michaelis constant, K_m = 1.2 ± 0.1 mM, was independently determined for the same substrate under the above experimental conditions. Data fitting to equations was carried out with a non-linear least squares procedure, as provided by program MINSQ (Micro Math Scientific Software, Salt Lake City, UT, USA).

Inhibition of the elastolytic activity of HLE

Elastin from bovine neck ligament (Sigma) was incubated with HLE with or without added inhibitors at various concentrations. 2.0 mg of finely powdered elastin were suspended in 400 µl of 57 mM sodium/potassium phosphate buffer (pH 7.40, I = 0.15), then 20 µl of inhibitor solution, diluted at the appropriate concentration in the same buffer, but containing 40% (v/v) DMSO, were added. The reaction was started by adding 10 µl of HLE prediluted in 0.1 M acetate buffer at pH 4.5 to give a final concentration of elastase active sites of 0.5 µM. The mixture was incubated at 37°C for exactly 120 min and reaction was stopped by adding 100 µl of 25% (w/v) trichloroacetic acid. After centrifugation, a 0.1-ml portion of clear supernatant was mixed to 3.0 ml of 0.2 M sodium borate buffer (pH 8.5), and 1.0 ml of a fluorescamine solution (0.15 mg/ml in acetone) was then added under vigorous stirring. The fluorescence of the labeled peptides was monitored with λ_{ex} = 390 nm and λ_{em} = 480 nm. The fluorescence obtained in the absence of inhibitor was taken as reference (100% activity). Appropriate blanks were run to take into account the fluorescence developed by elastin, enzyme and inhibitors alone. DMSO (final concentration 1.9%) did not interfere with the enzyme activity and the assay was linear with time up to 8 h in the absence of inhibitors.

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